STUDIES ON PHOSPHOPROTEINS

BY

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STUDIES ON PHOSPHOPROTEINS

INAUGURAL DISSERTATION

by

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By due permission of the King, to be publicly
discussed in the lecture hall of the Institute of Medical Chemistry,
May 21, 1955, at 10 a.m., for the degree of doctor of medicine.
In addition to this survey the papers I–X in the list of
references on page 3 will be publicly discussed.

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Studies on Phosphoproteins

This paper is a summary of results earlier published in the following articles:

I. Ågren, G., de Verdier, C.-H. and Glomset, J.: Crystalline Phosphoserine from Casein Hydrolysate, *Acta Chem. Scand.* 5 (1951) 324.

II. de Verdier, C.-H.: The Isolation of Phosphothreonine from Bovine Casein, *Ibid.* 7 (1953) 196.

III. de Verdier, C.-H.: Phosphorus-containing Amino Acids and Peptides from Acid Hydrolysates of Casein and Pepsin, *Ibid.* 8 (1954) 1302.

IV. de Verdier, C.-H.: On Phosphorus-containing Amino Acids and Peptides from Acid Hydrolysates of Casein, *Acta Soc. Med. Upsaliensis* 60 (1955) 48.

V. de Verdier, C.-H.: An Apparatus for the Registration of Radioactivity of Effluents from Chromatographic Columns, *Ibid.* 57 (1952) 393.

VI. de Verdier, C.-H. and Sjöberg, C. I.: An Automatic Conductivity Bridge for Chromatographic Analyses, *Acta Chem. Scand.* 8 (1954) 1161.

VII. Ågren, G., de Verdier, C.-H. and Glomset, J.: A Study of the Phosphorus-containing Proteins of Cells. I. The Isolation of Phosphoserine from the Liver Proteins of Calf and Rat, *Ibid.* 8 (1954) 503.

VIII. Ågren, G., de Verdier, C.-H. and Glomset, J.: A Study of the Phosphorus-containing Proteins of Cells. II. The Turnover Rate of P^{32}-labelled Phosphoserine of the Schneider Protein Residues of Several Rat Organs, *Ibid.* 8 (1954) 1570.

IX. Ågren, G., de Verdier, C.-H. and Glomset, J.: The Occurrence of Phosphoproteins in Bacteria, *Ibid.* 9 (1955) 196.

X. Ågren, G., de Verdier, C.-H. and Glomset, J.: On the Occurrence of a New Type of Phosphorylated, Ninhydrin-Positive Compound in Bacterial Protein, *Acta Soc. Med. Upsaliensis* 60 (1955) 30.

References to these papers will be made by using the Roman numerals given above.

It has long been held that phosphoproteins occur chiefly as nutrients for growing organisms, *viz.* in milk and in eggs, while they have been considered to be present in tissues only in small quantities (1). In the following phosphoproteins will be mainly classified according to their location.

Phosphoproteins in milk

Casein. Its heterogenity

Casein is the phosphorus-containing protein of milk. A brief survey of the casein literature can be found in (2). The protein is not homogeneous, but can be fractionated by different methods into α-, β- and γ-casein (3, 4). The fractions differ both in amino acid composition and in phosphorus content (5).

Already in 1933 Lipmann was able to isolate the barium and silver salts of SerP[1] from an acid hydrolysate of casein, thereby demonstrating that at least a part of the phosphorus in casein is very likely bound to the hydroxyl group of serine (6). This was verified by the isolation of free SerP from bovine casein (I). It also seemed possible, however, that the other β-hydroxy- amino acid, threonine, might occur in a phosphorylated form. The search for ThrP[1] led to the development of chromatographic methods, which have since been extended.

Chromatographic methods for phospho-amino acids

Two systems have chiefly been used:

1) Elution analysis with 0.01 N hydrochloric acid on sulfonic acid ion exchange columns (Dowex 50) (II). This is a modification of Stein and Moore's first system for the use of ion exchange resins in separating amino acids (7).

2) Elution analysis with N formic acid on quaternary ammonium base ion exchange resins (Dowex 1). In many cases the gradient elution principle has been used (VIII, VI, IV). This is the same system used by Hurlbert *et al.* for the separation of nucleotides (8).

In order to test the purity or obtain further fractionation of the phosphorus-containing amino acids and peptides, certain paper chromatographic systems have been worked out (VII).

Methods of registration used in the chromatographic analyses

In earlier experiments ninhydrin spot tests were used to indicate the phosphorylated amino acids. This method, however, was too insen-

[1] The following abbreviations are used: SerP = O-phosphoserine, ThrP = O-phosphothreonine. Amino acids according to (53).

sitive, and, moreover, it seemed preferable to employ a method which did not expend the substances which were to be isolated. Of the possible methods (V) conductivity measurement was considered to be among the most sensitive, since the electrical resistance of the elutrients is relatively high and—in the pH regions used—the substances markedly effect the concentration of hydronium ions. A sensitive but rather simple automatic conductivity bridge was therefore constructed (VI). Its use is demonstrated by the figures in (VI) and by figure 1 in (VII).

Subsequent experiments with tissue phosphoproteins were carried out in such a way that the phosphorus-containing amino acids became labelled with P^{32}. This was done partly to facilitate indication in the chromatographic separations, partly in order to study their role in phosphorus metabolism. The indication was often done by drying aliquots of the fractions on planchettes and measuring the radioactivity with more or less automatic Geiger-Müller counters (VII, 9). Here, as well, it has often been desirable to make the analyses completely automatic. This has been accomplished with the apparatus described in (V). In addition to the fact that the apparatus is completely automatic, it has the advantage that it gives a truer picture of the elution curve than the aliquot technique usually does. The volume of the spiral in which the radioactivity of the solution is measured is only about 1 ml and rarely do the values given by aliquot methods represent so small fractions. Moreover, all of the radioactivity is retained without extra precaution. The disadvantages of the method are: that it can only be used for γ- and hard β-rays emitters, and that it is usually less sensitive than the aliquot method.

In order to register the radioactivity of one dimensional paper chromatograms, an apparatus has been constructed (VIII), which is a modification of available commercial apparatuses.

Phosphorus-containing amino acids in casein

By partly utilizing the above chromatographic methods and modifications of them it was possible to isolate ThrP from acid hydrolysates of casein (10, 11). Certain data have been determined for both SerP and ThrP, such as elementary composition, R_F values in certain solvents, melting points, optical rotation, unit cell dimensions (I, II, IV) and absorption in the infrared (11).

The possibility of linkage between phosphorus and other amino acids in phosphoproteins cannot be excluded. A phosphopeptide from chicken liver contains cystein but no hydroxyamino acids (12). Free O-phosphohydroxylysine and argininephosphoric acid have been reported in animal tissue extracts (13, 14) and aspartyl phosphate in yeast extract (15).

In the ThrP isolation experiments, several fractions containing phosphopeptides were isolated. Earlier this type of peptide has mainly been obtained by proteolytic hydrolysis of casein and has then been called phosphopeptone.

Phosphopeptone

A perspicuous review of the phosphopeptone literature has been given by Mellander in (16). The amino acid composition has varied with the method of preparation (16, 17, 18), and chromatographic investigations by Ågren and Glomset (19) have shown that at least the phosphopeptone prepared according to Mellander is a complex mixture of more or less phosphorus-containing peptides.

Phosphopeptides from acid hydrolysates of casein

Levene and Hill were able to isolate a peptide from an acid hydrolysate of phosphopeptone, which consisted of SerP and glutamic acid (20). One year earlier a peptide containing the same amino acids had been isolated direct from phosphopeptone (21). The structure of the peptide from the acid hydrolysate was later determined to be SerP-glutamic acid (22).

As mentioned above, several fractions containing acid-resistant peptides were obtained during the attempts to isolate ThrP. Since they seemed to be smaller than those formed by enzymatic hydrolysis, they have been purified in order to obtain increased knowledge of the protein structure in the vicinity of the phosphate groups (III, IV). Certain experiments made in order to determine the optimum hydrolysis conditions for SerP have also been carried out (IV).

Very successful attempts to analyse the nature of the phosphorus linkage in casein have recently been published by Perlmann (23) and should be mentioned in this connection.

One cannot exclude the possibility that certain phosphate groups have migrated from amino to hydroxyl groups during the acid hydrolysis (24). Rearrangements within the peptides during hydrolysis with the rather dilute hydrochloric acid are also possible *via* piperazine formation (25, 26).

Phosphoproteins in eggs

Some results of studies by others of the phosphoproteins of eggs will be related here for purposes of comparison. Phosphoproteins can be classified according to their occurrence in egg yolk and egg white.

Phosphoproteins in egg yolk

A review of the proteins of egg yolk can be found in (27). An especially phosphorus-rich fraction, phosvitin, has been prepared, which has a phosphorus content of approximately 10 % (28). It also has a very high serine content (29). Lipmann and Levene were able to isolate SerP from a similar fraction before it was isolated from casein (30). The occurrence of ThrP in vitelline was suggested by Creyssel and Enselme (31) after it had been found in casein (10).

Phosphoproteins in egg white

Ovalbumin is the phosphorus-containing protein of egg white. It is a mixture of two components, one of which contains two phosphorus atoms, the other one phosphorus atom per molecule (32). Perlmann has studied ovalbumin using the same phosphatase technique which she used with casein (33). She considers that one of the bonds has the character of a phosphoamide linkage (-N-P-) while the other phosphorus atom is bound to a hydroxyl group (-O-P-). Recently, Flavin has published an excellent investigation of the composition of the peptide chain in the vicinity of the two phosphate groups (34). He employed partial hydrolysis with 11 N hydrochloric acid and then chromatographic systems similar to those described above, but complemented with zone electrophoresis.

Other extracellular phosphoproteins

Pepsin

Pepsin can be included in this group since it contains one atom of phosphorus per molecule. Pepsin has also been studied by Perlmann and Flavin with their respective methods of analysis. Perlmann considers the phosphorus to be bound in a diester linkage (35). Flavin has shown that one of the peptide sequences to which the phosphorus is bound has the structure: ThrSerPGlu (34). Independently of Flavin, the author was able to isolate crystalline SerP from pepsin (111).

A comparison of the phosphopeptides from acid hydrolysates of the different phosphoproteins shows that glutamic acid is commonly bound to SerP. Furthermore, SerP bound to alanine occurs both in casein and in ovalbumin. No phosphopeptide containing basic or aromatic amino acids has yet been isolated.

Phosphoproteins in animal tissue

Methods

Plimmer and Scott appear to be the first to have studied tissues with respect to phosphoproteins (1). They used the alkali lability of the phosphorus as a criterion in considering that it originated from phosphoproteins. Plimmer and Scott regarded the tissue content of phosphoproteins to be very low, with the possible exception of the pancreas. The system of fractionation of the phosphorus-containing compounds in tissues which was later developed by Schmidt and Thannhauser made use of the same principle with respect to the phosphoprotein phosphorus (36). Schneider developed another system of fractionation during the same year (37). In this method the phosphoproteins are obtained as a residue after several different extractions. The content of phosphoproteins in tissues has been reported to lie between 0.094 μg phosphorus *per* gram wet tissue for spleen and 0.021 μg P/g for heart (38).

After the phosphorus isotope, P^{32}, became available many studies of the metabolism have been made with it (*cf.* 39). Phosphorus was shown to reach the phosphoprotein fraction relatively quickly (38—40), and the phosphoprotein phosphorus was shown to have a high specific activity. But the results obtained with the methods of Schmidt and Thannhauser and of Schneider are not in agreement (38, 39), and since neither »phosphoprotein» fraction can be considered pure, we have therefore held that only after isolating a pure phosphoprotein or a characteristic phosphorus-containing segment from the protein molecule, a phosphoamino acid, should conclusions be drawn with respect to the phosphorus metabolism of phosphoproteins. The method is discussed in detail in (VII). However, it precludes the occurrence in tissues of phosphoproteins which yield phosphoamino acids on acid hydrolysis. By means of experiments on a preparative scale it has been possible to isolate crystalline SerP and ThrP from the Schneider rest protein fraction of calf and sheep liver. It must be considered very improbable that SerP is derived from lipides which have been incompletely extracted, and with respect to ThrP, it has as yet only been isolated from phosphoproteins.

Metabolism experiments

In another study (VIII) the uptake of labelled phosphorus into the phosphoproteins of various organs has been followed. The specific activity of the isolated SerP reaches its maximum approximately three hours after the subcutaneous injection of radioctive inorganic phosphate. The organ with the highest specific activity is the liver, while the lowest values are obtained in the brain. However, in the latter

organ the *quota* of the activity of the inorganic phosphorus released by the acid hydrolysis and that of SerP is the highest of all organs. If the distribution of radioactive phosphorus in the various organs is taken into consideration (41) the phosphoproteins of the brain do not appear to lack importance. Their function in nerve tissue has been touched upon in several studies (42, 43) in which the earlier methods for following phosphorus metabolism were used.

In one of our experiments the specific activity of SerP was compared with that of several phosphopeptides. No marked differences were found, however (VIII). At the time of maximal incorporation, the SerP in the phosphoproteins had a relatively high specific activity but the latter was exceeded by certain fractions from the cold trichloroacetic acid extract.

At approximately the same time that these methods were developed, Kennedy and Smith, using similar methods, studied the uptake of P^{32} in SerP of protein fractions from ascites tumor cells and liver mitochondria (44). They also were able to demonstrate the high specific activity of SerP.

The function of the phosphoproteins is unknown, although the rapid metabolism of the protein phosphorus indicated that SerP may be of special importance as an active center of the protein.

Several authors have studied the occurrence of phosphoprotein phosphatases in tissues (45—47). Recently, Kennedy and Burnett have been able to partially purify a protein phosphokinase (48). Continued investigations along this line may perhaps help to clarify phosphoprotein function. An improved knowledge of the structure of phosphoproteins is also to be desired. Some attempts to purify a phosphoprotein have been carried out (49). Flavin has shown that serine and SerP in ovalbumin have approximately the same specific activity following the injection of C^{14}-labelled glycine (34). It would be of interest to know whether this is also the case with respect to the active tissue phosphoproteins. The results of Kennedy *et al.* in investigations of tumor cells seem to indicate the importance of phosphoproteins in growth (44, 50).

Phosphoproteins in microorganisms

The possibility of a connection between phosphoprotein phosphorus metabolism and protein synthesis has been mentioned earlier. In order to study this problem further, *Lactobacillus casei* have been grown in a medium containing P^{32}-labelled phosphate. Investigations with *L. casei* of the growth-stimulating effect of phosphoamino acids and phosphopeptides have been made earlier in this laboratory (51). Experiments with yeast and labelled phosphate have been carried out by Juni

et al. (52). They fractionated the phosphorus-containing compounds by a modification of the Schmidt—Thannhauser procedure. Interpretation of their results, however, was complicated by the presence in many fractions of meta- and pyrophosphate. Juni *et al.* believe that much of the protein phosphorus occurs in the form of pyrophosphate.

In the last two articles (IX, X) the methods mentioned above have been used to study bacterial phosphoprotein. It has been possible to isolate a radioactive fraction from the protein, which has the same chromatographic behaviour as unlabelled SerP added as a carrier. However, much more of the radioactive phosphorus originally added to the medium can be found bound to another ninhydrin-positive substance which is neither serine nor threonine. Characteristic properties of this substance are described in (X); among these are several values for the specific activity of the compound after varying periods of growth. Attempts to determine the chemical structure of the compound are in progress, as well as attempts to correlate its specific activity with that of other phospho-compounds during the growth cycle.

Summary

Chromatographic methods have been worked out for the isolation of phosphorus-containing amino acids and peptides. In order to follow the course of the chromatography, automatic registration devices have been constructed based on conductivity and radioactivity measurement.

It has been possible to isolate phosphothreonine from acid hydrolysates of casein and thereby to show that phosphoprotein phosphorus can also be bound to this amino acid. In addition to phosphothreonine, several phosphopeptides have been isolated.

Independently of Flavin (34), crystalline phosphoserine has been isolated from pepsin.

The methods developed have also been used to study phosphoproteins in animal tissue and in bacteria. Crystalline phosphoserine and phosphothreonine have been isolated from liver protein. In isotope studies the rapid metabolism of the serine-bound phosphorus in phosphoproteins has been demonstrated in several organs. Phosphoserine also appears to occur in bacterial protein, while the major part of the phosphorus is bound to an as yet unknown ninhydrin-positive substance.

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